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14. ABSTRACT

Cyclooxygenase-2 (COX-2) and its product PGE2 are known to promote tumor growth and to enhance the penetration of cancer cells into adjacent tissues. Thus, knowing how the activity of COX2 is regulated at the cellular level has implications for breast cancer therapeutic strategies. The goal is to unravel a new molecular mechanism for regulating the activity of COX-2. In plasma membranes, arachidonic acid (AA) is released by phospholipase A2 (PLA2). COX then converts AA to prostaglandins. The activity of PLA2 is known to vary with cholesterol content in an alternating manner, showing a local minimum at critical sterol mole fractions for maximal superlattice formation. In this study, the cholesterol content in MCF-7 human breast cancer cells was depleted by using methyl-beta-cyclodextrin. A biphasic change in COX-2 activity was observed at certain cell cholesterol content Cr.

15. SUBJECT TERMS
Membranes; cyclooxygenase; cancer invasion; MCF-7 cells; cellular signaling

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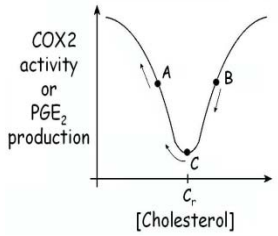
INTRODUCTION

Objectives. Cyclooxygenase-2 (COX2) and its product PGE_2 (a prostaglandin) are known to increase both angiogenesis and resistance to apoptosis (promoting tumor growth) and to enhance the penetration of cancer cells into adjacent tissues (causing metastasis). Thus, knowing how the activity of COX2 is regulated at the cellular level has implications for breast cancer therapeutic strategies. The **goals** of this proposed research are to unravel a new molecular mechanism for regulating the activity of COX2, to provide evidence that this mechanism may regulate cell proliferation in cultured breast cancer cells, and to use this newly revealed mechanism to develop novel liposomal drug formulations to treat breast cancer cells.

Hypothesis. Arachidonic acid (AA) is released from the plasma membrane by phospholipase A2 (PLA2). Cyclooxygenase (COX) converts AA to prostaglandins (e.g., PGE_2). The activity of PLA2 is known to vary with membrane cholesterol content in a nalternating manner, showing a local minimum at critical sterol mole fractions (C_r) for maximal superlattice formation. Hence, it is logical to **hypothesize** that the activity of COX (including the isoform COX2) also varies with cholesterol content in a biphasic manner, showing a minimum at C_r (see diagram). The biphasic change in COX2 activity should occur within a narrow range of cholesterol content near C_r. As a result, C_r serves as a bio-switch that can regulate the production of PGE_2. When the cholesterol content is near C_r, the COX2 activity and the PGE_2 production are low. When the cholesterol content deviates significantly from C_r (either >> C_r or << C_r), the COX2 activity, thus the production of PGE_2, becomes high. As such, the cholesterol content near C_r serves as a fine-tuning mechanism to regulate COX2 activity and PGE_2 production, and consequently, cancer cell growth and metastasis. Using the same sterol superlattice concept, one can design new liposomal drug formations for optimum anti-cancer activities.



Innovation. This research may reveal a new role of cholesterol in breast cancer and a new strategy to treat breast cancer. According to the above-mentioned hypothesis, the initial membrane cholesterol content in cells will determine how a decrease in cholesterol content would affect COX2 activity. For example, if the initial membrane sterol content is at A or C (see the diagram), a decrease in cholesterol will bring up the COX2 activity. If the initial sterol content is at B, a decrease in cholesterol will decrease the cellular activity (until it reaches C). This implies that while one could take cholesterol lowering drugs, such as statins, to reduce serum cholesterol and thereby lower cardiovascular risks, the decrease in cholesterol might also greatly increase PGE_2 production, causing an increase in tumor growth and cancer invasion. In fact, animal studies showed that long-term statin use may be carcinogenic and epidemiological evidence suggests that some statins increase the incidence of breast cancer. This research may provide partial explanations to these puzzling findings. Moreover, the biophysical principles revealed in this study may lead to new strategies of using liposomal drug to treat breast cancer.

BODY

Cells. The human MCF-7 breast cancer cells (from ATCC) were grown in Complete Growth Medium which includes Dulbecco's Modification of Eagle's Medium (DMEM 1x, with 4.5g/L glucose, L-glucose & sodium pyruvate) supplemented with 10% Fetal Bovine Serum (FBS, BioWhittaker, Walkersville, MD) and 1% penicillin/streptomycin at 37°C with 5% CO_2 atmosphere. Cell culture prior to confluence were harvested and used for our experiments.

Modification of cell cholesterol content. For cholesterol depletion, cells were incubated in serum-free medium with different amounts of methyl-β-cyclodextrin (MβCD) for various times at 37°C. Following incubation with cells, MβCD was removed by washing with PBS buffer.

COX-2 activity measurements. The activity of exogenously added human recombinant COX-2 was triggered by addition of phospholipase A2 (PLA2) to cells and monitored at 37°C using the COX-2 activity kit from Cayman Chemicals. The COX activity assay kit measures the peroxidase activity of COX. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm using a microplate reader. The kit includes isozyme-specific inhibitors for distinguishing COX-2 activity from COX-1 activity. The COX-2 activity will be calculated using the TMPD extinction coefficient of 0.00826 μM^{-1}. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmole of TMPD per minute at 25°C. It takes 2 molecules of TMPD to reduce PGG_2 to PGH_2. The COX-2 activity was plotted as a function of cholesterol content relative to total membrane lipids in the cell or a function of [MβCD]. Lipids in cells were extracted by a chloroform-methanol solvent mixture (2:1, v/v). The chloroform layer was taken out for cholesterol and phospholipid determinations.

Cell proliferation assay. MCF-7 cell proliferation was monitored by using the CyQUANT Cell Proliferation Assay kit (Invitrogen). The fluorescence readings from this assay were made on a Molecular Device microplate reader (Spectra Max M5). Fluorescence intensities were converted to cell numbers using a standard curve.

Liposomal CA4P anticancer drug. The antivascular prodrug combretastatin A4 disodium-phosphate (CA4P) was incorporated into the inner aqueous compartment of the liposomes made of 1-palmitoyl-2-oloeyl-phosphatidylcholine (POPC) and cholesterol. The cholesterol content in the liposomes was changed with 0.4 mol% increments, and each sample set was centered around one particular critical sterol mole fraction (C_r) for maximal superlattice formation. Free CA4P was removed by gel filtration. Liposomal CA4P was incubated with cultured MCF-7 cells for 30 min and then cell proliferation was determined using the CyQUANT assay kit.

Results.

Figure 1 (see Supporting Data) demonstrates that the COX-2 activity in MCF-7 cells varies with cell cholesterol content in a non-monotonic manner. The MCF-7 cells were treated with 0.0, 0.25, 0.75, 1.0, and 1.75% MβCD for one hour. The cells were then washed twice to remove MβCD. Two sets of samples were tested. The results were reproducible and showed a trend of biphasic change in COX-2 activity with cell cholesterol content. The %MβCD is inversely proportional to cell membrane cholesterol. These data provide supporting evidence that the proposed experiment is feasible and that our hypothesis is reasonable and testable. These results suggest that membrane cholesterol content does affect COX2 activity and that the cholesterol dependence of COX-2 activity is likely to be multi-biphasic.

The detection of multiple biphasic changes at particular sterol mole fractions could be a sign of possible sterol superlattice formation. However, to demonstrate the cholesterol dependence of COX-2 activity is multi-biphasic, we need to extend the study to a larger cholesterol mole fraction range using smaller cholesterol increments. A real challenge is whether small cholesterol increments (e.g., 1 mol%) in cells could be readily created or measured. In a separate study, we were able to reliably create ~ 1-2 mol% cholesterol increments in Chinese Hamster Ovary (CHO) cells and demonstrated that the determinations of cholesterol content relative to phospholipids, in most cases, were accurate to 0.2-0.7 mol%. Using these experimental conditions, we were able to demonstrate a biphasic change in lipid raft density isolated from CHO cells at, at least, two cholesterol mole fractions, a sign consistent with the concept of sterol superlattice formation. Thus, we are confident that similar cholesterol mole fraction increments can be achieved in MCF-7 cells. In the future experiments, we will extend our study of MCF-7 (e.g., the data shown in Figure 1) to a much larger cholesterol range.

Liposomes have been used as anti-breast cancer drug carriers for targeted delivery. In the no-cost-extension period (9/08-8/09), we have investigated how sterol lateral organization of liposomal CA 4P can affect the MCF-7 cell proliferation and the rate of CA4P release from the liposomes. The preliminary results showed that the cell proliferation rate varies with cholesterol content in the liposomal drug in a biphasic manner, displaying a minimum at 22.7 mol% cholesterol in POPC. This sterol mole fraction is close to that theoretically predicted (22.2 mol%). In a separate study, we found that at C_r (22.2 mol% cholesterol) the leakage of CA4P is faster than at non-critical mole fractions (Figure 2). The drug release data (Figure 2) explain why cell proliferation is lower at C_r. Although cholesterol superlattice domains have tighter lipid packing, the defects that are produced in the interfaces between regular and irregular domains enhance the overall membrane permeability. Therefore the extent of cholesterol superlattice can be used to modulate the release of encapsulated

drugs. This property can be employed to optimize the breast cancer treatment by liposomal CA4P.

Significance. If the hypothesis is correct, then reaching a local minimum in COX2 activity at C_r may be a wise therapeutic goal. **This concept could be used to develop a new treatment strategy for the reduction of breast cancer tumor growth and metastasis, which could then be used in combination with existing treatments.** The idea is that when the cell membrane cholesterol level deviates appreciably from those values which produce minimum COX2 activity, the cells are more prone to cancer proliferation and invasion due to higher levels of PGE_2 production. In this case, one should manipulate the cholesterol content in cell plasma membrane by locally applying cholesterol lowering or enrichment drugs to the troubled tissues via targeted drug delivery technology. The same rationale can be applied to develop new strategies to optimize the anticancer activity of CA4P and other anticancer drugs.

A list of personnel receiving payments:

Berenice Venegas, Associate Scientist
Michelle Olsher, Associate Scientist
Parkson Chong, PI

KEY RESEARCH ACCOMPLISHMENTS

- COX-2 activity varies strongly with cell cholesterol content.
- COX-2 activity varies with cell membrane cholesterol content in a non-monotonic manner.
- There is a sign that COX-2 activity in MCF-7 breast cancer cells varies with cell cholesterol content in a multiple biphasic manner, in agreement with the concept of sterol superlattice.
- The anticancer activity of the antivascular drug CA4P varies with liposomal cholesterol content in accordance with the physical principles of sterol superlattice formation

REPORTABLE OUTCOMES

Meeting abstracts

Chong, P. L.-G., Olsher, M., Venegas, B., Zhu, W., and Tran, S. (2008) A New Mechanism for Modulating the Activity of the Cancer Invasion Promoter Cyclooxygenase-2. A poster presented at the Era of Hope meeting in Baltimore, Maryland, June, 2008.

Zhu, W., Olsher, M., Venegas, B., Tran, S. and Chong, P. L.-G. (2009) Role of Membrane Cholesterol Content on the Activity of Cyclooxygenase-2 (COX-2) in

MCF-7 Human Breast Cancer Cells. A poster presented at the Biophysical Society Meeting in Boston, MA, March 2009.

Venegas, B. and Chong, P.L.-G. (2010) Drug Release from Liposomes Can Be Modulated by the Extent of Cholesterol Superlattice in the Lipid Membrane. A poster to be presented at the Biophysical Society Meeting in San Francisco, CA, February 2010.

CONCLUSION

We were able to modify the cholesterol content in MCF-7 human breast cancer cells and demonstrate that the COX-2 activity varies with cholesterol content in a non-monotonic manner. This result provides a good starting point to eventually demonstrate that the COX-2 signaling pathway is regulated by the extent of sterol superlattice. The extent of sterol superlattice in liposomal drug formulations is an important factor of the drug's anticancer activity.

APPENDICES

Three meeting abstracts (see above)

1. Chong, P. L.-G., Olsher, M., Venegas, B., Zhu, W., and Tran, S. (2008) A New Mechanism for Modulating the Activity of the Cancer Invasion Promoter Cyclooxygenase-2. A poster presented at the Era of Hope meeting in Baltimore, Maryland, June, 2008.

Background and objectives:

Cyclooxygenase-2 (COX2) and i ts product P GE_2 (a pr ostaglandin) are known t o promote t umor g rowth by i ncreasing ang iogenesis, metastasis and resistance to apoptosis. Thus, knowing how the activity of COX2 is regulated at the ce llular l evel has implications for br east ca ncer t herapeutic strategies. T he goals of this proposed research are to unravel a new molecular mechanism for regulating the activity of COX2 and to provide evidence that this mechanism may regulate cell proliferation in cultured breast cancer cells.

Arachidonic acid (AA) is released from the plasma membrane by the action of phospholipase A2 (PLA_2); COX2 then converts AA to prostaglandins. The activity of PLA_2 is known to vary with membrane cholesterol content in a biphasic manner, showing a local minimum at critical sterol mole fractions (C_r) for maximal superlattice formation. We hypothesize that the activity of COX2 will also vary in an alternating manner, allowing plasma membrane cholesterol content to function as a bioswitch to regulate the production of PGE_2. When the cholesterol content is near C_r, the COX2 activity and the PGE_2 production are low. When the cholesterol content deviates significantly from C_r (either >> C_r or << C_r), the COX2 activity, thus the production of PGE_2, becomes high. As such, the cholesterol content near C_r serves as a fine-tuning mechanism to regulate COX2 activity and PGE_2 production, and consequently, cancer cell growth and metastasis.

Description:

Using methyl-beta-cyclodextrin (mβCD), we are altering cholesterol levels in very small increments (~1 mol%) in the plasma membranes of MCF-7 cells. After cholesterol depletion, COX2 activity levels can be measured using the COX2 activity kit (Cayman Chemicals). The COX2 activity will be then plotted as a function of cholesterol content relative to total membrane lipids in the plasma membrane. Total membrane lipids will be determined by capillary electrophoresis and mass spectrometry. The extent of cancer cell proliferation will be determined fluorometrically using a CyQUANT assay kit from Molecular Probes on cells with varying cholesterol content alterations. All the experiments will be performed in triplicate.

Results:

We are currently assessing the results of preliminary COX2 assays. Using methyl-beta-cyclodextrein (mβCD), we are altering the cholesterol levels in the plasma membranes of MCF-7 cells in very small increments over a wide cholesterol concentration range. After cholesterol depletion, COX2 activity levels

can be measured in different cell cultures with varying membrane cholesterol concentrations.

Conclusions:

If the hypothesis is correct, then reaching a local minimum in COX2 activity at C_r may be a wise therapeutic goal. This concept could be used to develop a new treatment strategy for the reduction of breast cancer tumor growth and metastasis, which could then be used in combination with existing treatments. The idea is that when the membrane cholesterol level deviates appreciably from those values which produce minimum COX2 activity, cells are more prone to cancer proliferation and invasion due to higher levels of PGE_2 production. In this case, one should manipulate the cholesterol content in cell plasma membrane by locally applying cholesterol lowering or enrichment drugs to the troubled tissues via targeted drug delivery technology.

2. Zhu, W., Olsher, M., Venegas, B., Tran, S. and Chong, P. L.-G. (2009) Role of Membrane Cholesterol Content on the Activity of Cyclooxygenase-2 (COX-2) in MCF-7 Human Breast Cancer Cells. A poster to be presented at the Biophysical Society Meeting in Boston, MA, March 2009.

Cyclooxygenase-2 (COX-2) and its product PGE2 are known to increase both angiogenesis and resistance to apoptosis (promoting tumor growth) and to enhance the penetration of cancer cells into adjacent tissues (causing metastasis). Thus, knowing how the activity of COX2 is regulated at the cellular level has implications for breast cancer therapeutic strategies. The goal of this research is to unravel a new molecular mechanism for regulating the activity of COX-2. The proposed molecular mechanism may be elucidated by using the sterol superlattice model. In plasma membranes, arachidonic acid (AA) is released by phospholipase A2 (PLA2). Cyclooxygenase (COX) then converts AA to prostaglandins (e.g., PGE2). The activity of PLA2 is known to vary with membrane cholesterol content in a nalternating manner, showing a local minimum at critical sterol mole fractions (C_r) for maximal superlattice formation. Hence, it is logical to hypothesize that the activity of COX (including the isoform COX-2) also varies with cholesterol content in a biphasic manner. In this study, the cholesterol content in MCF-7 human breast cancer cells was depleted by using methyl-beta-cyclodextrin. A biphasic change in COX-2 activity was observed at certain cell cholesterol content C_{rcell}. The cholesterol content near C_{rcell} could serve as a fine-tuning mechanism to regulate COX-2 activity and PGE2 production, and consequently, cancer cell growth and metastasis.

3. Venegas, B. and Chong, P.L.-G. (2010) Drug Release from Liposomes Can Be Modulated by the Extent of Cholesterol Superlattice in the Lipid Membrane. A poster to be presented at the Biophysical Society Meeting in San Francisco, CA, February 2010.

Liposomes have been used as drug carriers for targeted delivery. Much attention has been paid to the stealth properties of the liposome in order to avoid the

immune system and have a prolonged circulation time. An aspect in the liposome design as a drug delivery system that has been relegated is the passive drug leakage from liposomes. In this work we investigated how lateral distribution of lipids in membranes can affect the overall leakage of an entrapped drug. For this study we used the antivascular drug Combretastatin A4 disodium-phosphate (CA4P) that has entered clinical trials for the treatment of a variety of cancers and is naturally fluorescent. CA4P was encapsulated in liposomes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/cholesterol at a quenching concentration (30 mM). Cholesterol content was varied in steps of 0.4 mol% in a range of concentrations covering the theoretically predicted critical mole fractions (C_r, e.g., 20.0, 22.2, 25.0, 33.3, 40.0 and 50.0 mol%) for maximal sterol superlattice formation. The non-encapsulated CA4P was removed by size exclusion column chromatography. The leakage was followed in real time by exciting CA4P at 328nm and reading fluorescence at 400nm. The results obtained show that at C_r the leakage of CA4P is faster than at non-critical mole fractions. Although cholesterol superlattice domains have tighter lipid packing, the defects that are produced in the interfaces between regular and irregular domains enhance the overall membrane permeability. Therefore the extent of cholesterol superlattice can be used to modulate the release of encapsulated drugs. Ongoing work is aimed to observe how this modulation will affect CA4P treatment using endothelial and mammary cancer cell lines. (supported by DOD breast cancer program)

SUPPORTING DATA

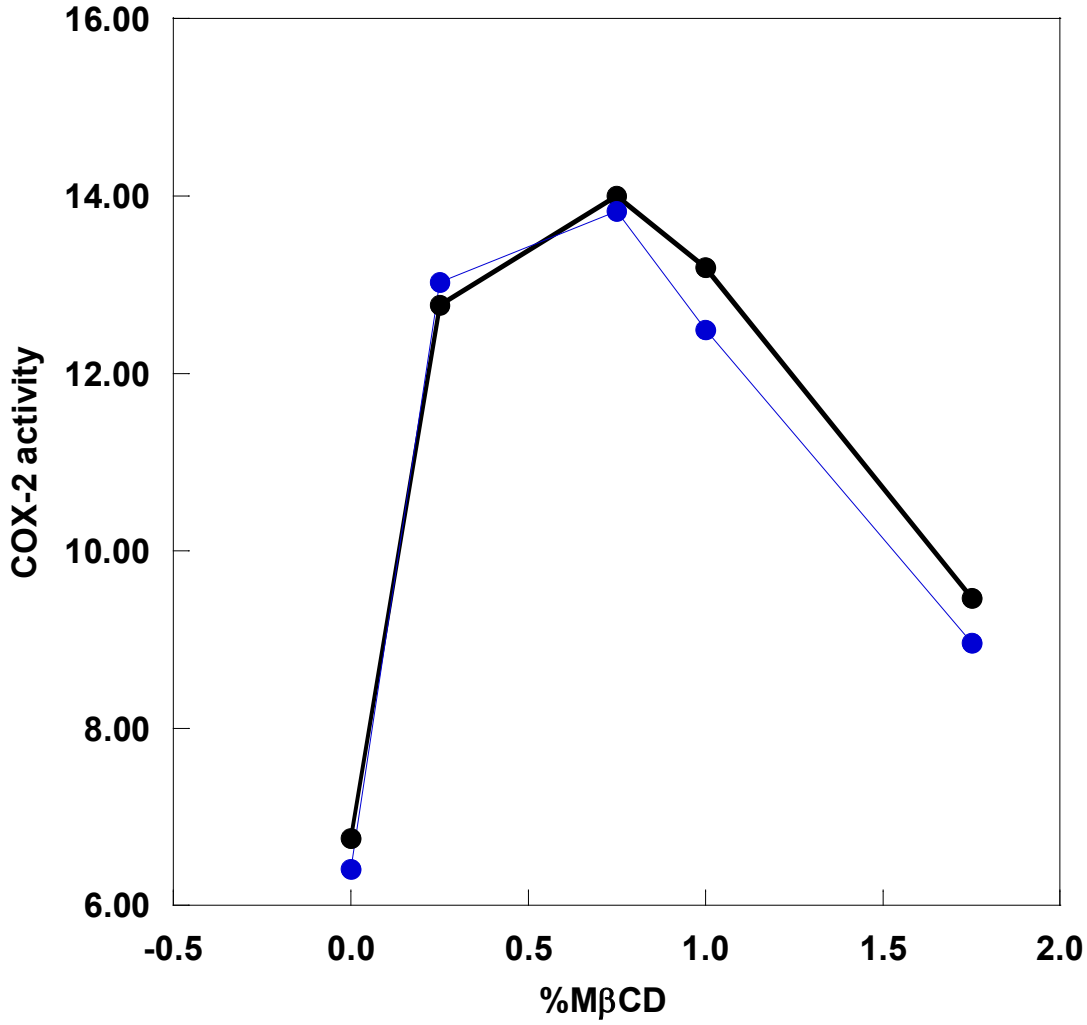


Figure 1. Plot of COX-2 activity versus weight percent of MβCD used to treat MCF-7 cells. Activity was measured at 24^{o}C.

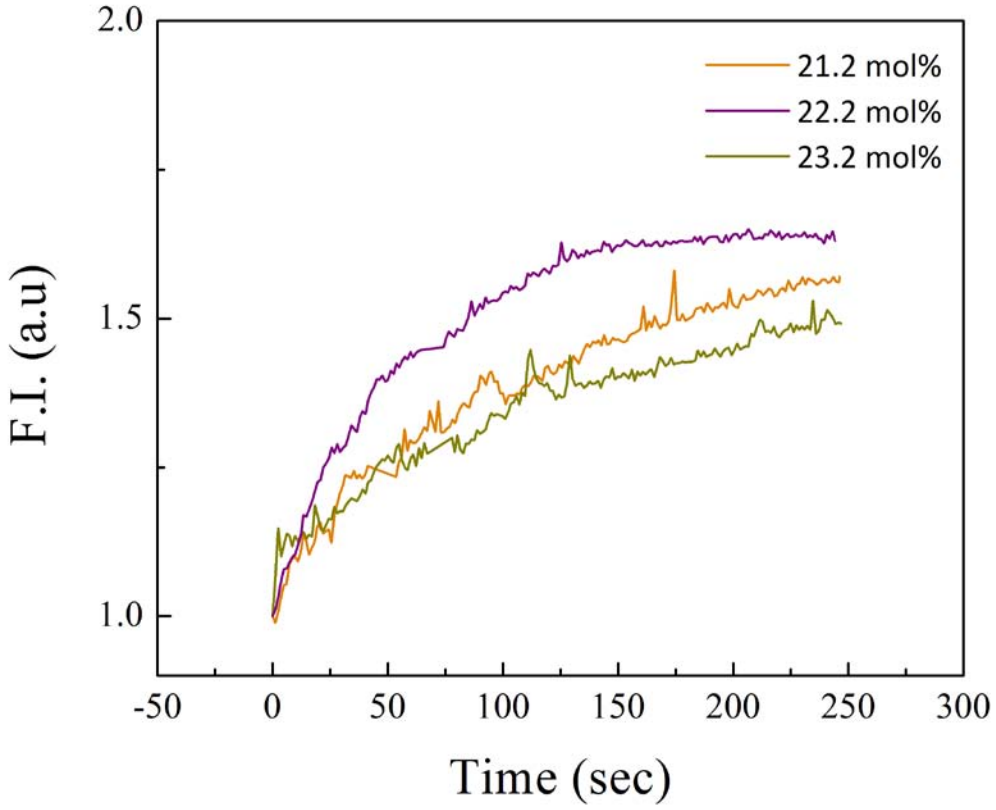


Figure 2. Effect of cholesterol mole fraction on the leakage of CA4P from POPC/cholesterol unilamellar vesicles. The initial leakage is slower at C_r (e.g., 22.2 mol% cholesterol) and faster at non-C_r (e.g., 21.2 and 23.2 mol% cholesterol). F.I. is the fluorescence intensity of CA4P.